

## **EXHIBIT 2**

## BAG-1: A Novel Biomarker Predicting Long-Term Survival in Early-Stage Breast Cancer

By Bruce C. Turner, Stanislaw Krajewski, Maryla Krajewska, Shinichi Takayama, Andrew A. Gumbs, Darryl Carter, Timothy R. Rebbeck, Bruce G. Haffty, and John C. Reed

**Purpose:** Among women with early-stage breast cancer treated with lumpectomy and radiation therapy, 30% to 40% will develop metastatic disease, which is often fatal. A need exists therefore for biomarkers that distinguish patients at high risk of relapse. We performed a retrospective correlative analysis of BAG-1 protein expression in breast tumors derived from a cohort of early-stage breast cancer patients.

**Patients and Methods:** Archival paraffin blocks from 122 women with stages I to II breast cancer treated with lumpectomy and radiation therapy (median follow-up, 12.1 years) were analyzed by immunohistochemical methods using monoclonal antibodies recognizing BAG-1 and other biomarkers, including Bcl-2, estrogen receptor, progesterone receptor, p53, and HER2/Neu. Immunostaining data were correlated with distant metastasis-free survival (DMFS) and overall survival (OS).

**Results:** Cytosolic immunostaining for BAG-1 was upregulated in 79 (65%) of 122 invasive breast cancers ( $P < .001$ ) compared with normal breast. Elevated BAG-1 was significantly associated with longer DMFS and OS, overall (stages I and II) and in node-negative (stage I only) patients, on the basis of univariate and multivariate analyses (DMFS,  $P = .005$ ; OS,  $P = .01$ , in multivariate analysis of all patients; DMFS,  $P = .005$ ; OS,  $P = .001$ , in multivariate analysis of node-negative patients). All other biomarkers failed to reach statistical significance in multivariate analysis. Clinical stage was an independent predictor of OS ( $P = .04$ ) and DMFS ( $P = .02$ ).

**Conclusion:** These findings provide preliminary evidence that BAG-1 represents a potential marker of improved survival in early-stage breast cancer patients, independent of the status of axillary lymph nodes.

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ONE OF EVERY nine women currently develops breast cancer. Among women with early-stage breast cancers treated with lumpectomy and local radiotherapy, 10% to 20% will experience local recurrences and 30% to 40% will develop distant metastatic disease, which is often fatal.<sup>1-6</sup> A need exists for identifying prognostic markers that accurately predict long-term outcome in these patients, thus permitting rational choices among therapeutic options such as adjuvant chemotherapy, surgical resection, radiation therapy, hormonal therapy, and application of newly discovered experimental therapeutics. This is especially important for women with axillary lymph node-negative breast cancer, where the use of systemic chemotherapy and hormonal therapy

remains controversial, and thus, decisions regarding clinical management are especially problematic.<sup>7,8</sup>

Several biomarkers have been shown to provide prognostic information for patients with breast cancer. Overexpression of the HER2/Neu oncoprotein, for example, has been shown to predict breast cancer patients at risk for metastatic disease and shorter survival in some studies.<sup>9-11</sup> The overexpression of the HER2/Neu protein is also used to identify patients that may be candidates for treatment with the humanized monoclonal antibody to HER2/Neu, trastuzumab (Herceptin; Genentech, South San Francisco, CA).<sup>12</sup> Somatic mutations that inactivate the p53 gene, with resulting overexpression of p53 protein, have been identified as predictors of poor prognosis in many subgroups of breast cancer patients.<sup>13-16</sup> Conversely, the expression of estrogen receptor (ER) and the Bcl-2 protein have been associated with favorable outcome in early-stage breast cancer.<sup>17-19</sup> However, among the many biomarkers studied, only the use of ER, progesterone receptor (PR) status, and perhaps immunostaining to detect mutant p53 provides sufficiently reliable information for clinical decision making.

BAG-1 is a multifunctional protein that contains a domain that binds tightly to Heat Shock 70-kd (Hsp70) family molecular chaperones and appears to modulate stress-responses through interactions with a variety of intracellular proteins, thereby regulating diverse cellular processes relevant to cancer, including cell division, cell survival, and cell migration.<sup>20-27</sup> Nuclear and cytosolic isoforms of the

From Thomas Jefferson University, Department of Radiation Oncology and Kimmel Cancer Center; University of Pennsylvania School of Medicine, Department of Epidemiology and Biostatistics, Philadelphia, PA; The Burnham Institute, La Jolla, CA; and Yale University School of Medicine, Departments of Therapeutic Radiology and Pathology, New Haven, CT.

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Address reprint requests to John C. Reed, MD, PhD, The Burnham Institute, 10901 North Torrey Pines Rd, La Jolla, CA 92037; email: jreed@burnham.org.

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BAG-1 protein are produced from a common mRNA by use of alternative translation initiation sites.<sup>28-30</sup> Interestingly, longer nuclear isoforms of BAG-1 reportedly bind to several types of steroid hormone receptors, including ER, and modulate the activities of some of these transcription factors.<sup>30-32</sup> The expression and prognostic significance of BAG-1 expression in breast cancers has not been fully examined, but a recent study suggests that BAG-1 may be unregulated in invasive breast cancer and associated with compromised survival.<sup>33</sup> Using monoclonal antibodies that allow specific detection of human BAG-1 proteins, we have examined the expression patterns of the BAG-1 protein in normal mammary tissues and invasive breast cancers, revealing associations of BAG-1 with patient survival in early-stage breast cancer patients.

## PATIENTS AND METHODS

### Patient Population

We identified 122 early-stage (stage I;  $n = 78$ ; 64%) (stage II;  $n = 44$ ; 36%) breast cancer patients treated at Yale University School of Medicine between 1978 and 1993, representing 20% of the breast cancer database registry at that time and for whom the primary paraffin tumor blocks were available for analysis (Table 1). Patients were excluded from molecular analysis if the breast tumor blocks were unavailable. Staging was performed according to the American Joint

Committee on Cancer, and all patients underwent bone scans to evaluate for metastatic disease. All patients were treated by lumpectomy, with or without axillary dissection, followed by radiation therapy to the intact breast using a median dose of 48 Gy followed by an electron boost to the lumpectomy site to yield a total median dose of 64 Gy. Of the 122 patients, 18 (15%) were treated with adjuvant systemic chemotherapy and 20 (16%) were treated with tamoxifen therapy (Table 1). Treatment was verified by chart review. Of the cohort, 63 of 122 (52%) underwent axillary lymph node dissection with a minimum of six sampled lymph nodes (Table 1). Lymph node biopsies were histologically negative for cancer in 49 (78%) of 63 women, whereas 14 (22%) of 63 had histologic evidence of metastatic spread to axillary lymph nodes (Table 1). The median follow-up for the patient cohort was 12.1 years. The start of the follow-up interval commenced at time of diagnosis, and failure required clinical or radiographic evidence of metastatic disease. Survival was ascertained from the breast cancer database that is updated yearly and maintained by full-time staff. Overall survival (OS) was defined as the time of diagnosis to last follow-up date or time of death for all patients, distant metastasis-free survival (DMFS) as time of diagnosis to development of first evidence of clinical or radiographic metastatic disease, and cause-specific survival (CSS) as the time of diagnosis to death with breast cancer. All patients with deaths not related to breast cancer were included up until the time of their death, after which they were excluded. We defined follow-up period as the time between the beginning of follow-up at diagnosis and a failure event (OS, DMFS, or CSS). If none of these events occurred, individuals were censored at the time of their death from other causes or at the end of the follow-up period. The study was approved by the Human Investigations Committee at the Yale University School of Medicine.

### Immunohistochemical Analysis of BAG-1 and Other Proteins

Paraffin-embedded blocks containing primary tumor specimens fixed in 10% neutral-buffered formalin were evaluated for invasive ductal carcinoma by hematoxylin-eosin staining and processed for immunohistochemical analysis as described previously.<sup>28,34</sup> Tissue sections were deparaffinized in xylene, rehydrated in ethanol, rehydrated with water, and washed in 1% phosphate-buffered saline. After blocking sections with 10% (vol/vol) goat serum, primary antibodies were applied to slides and incubated overnight, using a Dako Universal Staining System automated immunostainer (Dako, Carpinteria, CA). These primary antibodies included a mouse monoclonal antibody specific for human BAG-1 (KS6C8; 0.1% vol/vol ascites) and a rabbit polyclonal for Bcl-2, previously developed in our laboratory.<sup>28,35</sup> The specificity of these antibody reagents has been demonstrated by immunoblotting, immunoprecipitation, peptide-competition, and other methods.<sup>28,35</sup> After washing, the slides were incubated for 30 minutes with either antimouse or antirabbit biotinylated antibody (1:500 vol: vol) followed by either an avidin-biotin HRPase complex reagent (Vector Laboratories, Burlingame, CA), or the Envision-Plus-HRP system (Dako), with diaminobenzidine-based colorimetric detection. Among the other proteins analyzed using immunohistochemistry were several biomarkers previously suggested to provide prognostic information for breast cancer patients. These other biomarkers were detected using monoclonal antibodies to ER and PR (Abbott Laboratories, Abbott Park, IL) using the previously described ER immunohistochemical assay method, monoclonal antibody to HER2/Neu (1:1000 dilution; Dako), and a monoclonal antibody (DO7; 1:750 vol:vol) that recognizes mutant p53 (Oncogene; Cambridge, MA). We also stained

Table 1. Characteristics of Breast Cancer Patients and Tumors

Data	No.	%
Total patients	122	NA
Age at presentation, years		NA
Mean	54	
Range	28-82	
Infiltrating ductal cancer, n	109	89
Infiltrating lobular cancer, n	9	8
Infiltrating medullary cancer, n	4	3
Follow-up, years		NA
Median	12.1	
Range	2-18	
Stage, n		
I/II	122	100
I	78	64
II	44	36
Mean pathologic size, cm	1.7	NA
Axillary dissection, n	63	52
Lymph nodes, n		
Positive	14	12
Negative	49	40
Unknown	59	48
ER positive, n	50	41
PR positive, n	45	37
Adjuvant chemotherapy, n	18	15
Adjuvant tamoxifen, n	20	16

NOTE. Metastatic disease indicates patients who developed clinical or radiographic evidence of detectable metastatic disease.

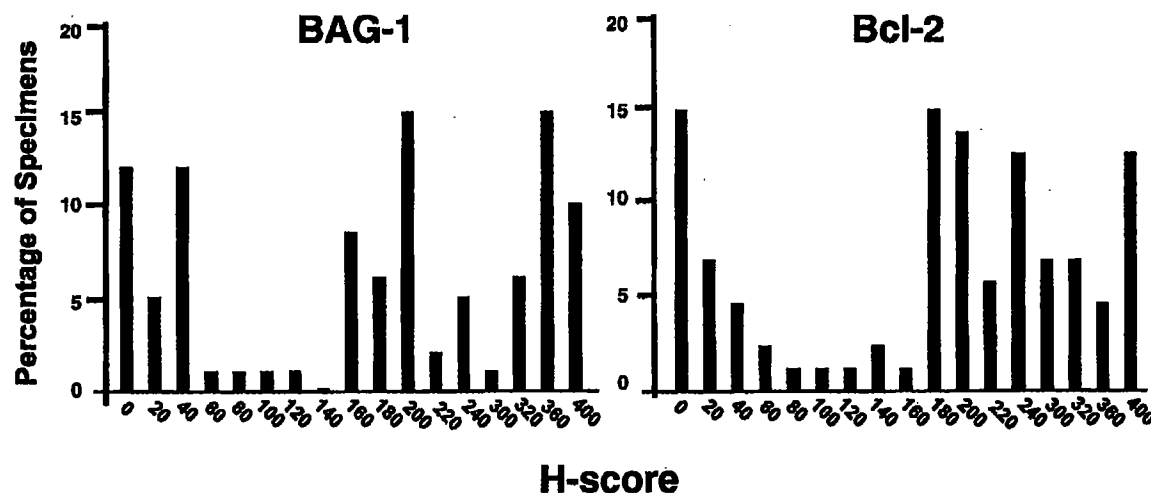


Fig 1. Distribution of BAG-1 and Bcl-2 immunostaining data in invasive breast cancers. The H-score data for invasive breast cancers are presented with H-score on the abscissa and the number of specimens having a particular H-score on the ordinate.

the cohort with an additional monoclonal antibody to ER (6F11; Novocastra Laboratories, Newcastle, United Kingdom, Newcastle-Upon-Tyne, United Kingdom).<sup>17</sup> Immunoscoring was performed separately for both invasive and benign components. The intensity of immunostaining on each slide was rated on a four-point scale: 0, none; 1+, light; 2+, moderate; 3+, heavy; and 4+, intense. The percentage of immunopositive tumor cells was determined by counting a minimum of 200 cells from at least three representative high-power fields. H-scores were then calculated as the product of intensity (0 to 4) X distribution (0% to 100%), with H-scores ranging from 0 to 400. Two tumor sections from each tumor specimen were immunostained and scored separately to minimize any effects of immunohistochemical heterogeneity. However, BAG-1 immunostaining patterns did not significantly differ among pairs of sections from the same breast tumors (data not shown). The pathologist (D.C.) scoring the stained specimens was blinded to the clinical histories of the patients. All samples were reread in a blinded fashion by the pathologist, and the mean H-score of both tumor sections was determined. To set cutoffs for dichotomization of data into high (positive) and low (negative) expression groups, the mean H-score data for the entire data set were displayed as bar-histograms (Fig 1), with H-score on the x-axis and the number of patient samples having a given H-score on the y-axis. A H-score  $\geq 150$  was determined by this approach to be appropriate for use as a cutoff for BAG-1 positivity. Individual H-scores were also determined for Bcl-2 (H-score  $> 180$ ), HER2/Neu (H-score  $\geq 25$ ), p53 (H-score  $\geq 50$ ), ER (H-score  $\geq 75$ ), and PR (H-score  $\geq 75$ ), as previously described.<sup>16,17,36</sup>

#### Statistical Analysis

All patient data, including clinical, pathologic, and outcome measures were entered into a computerized database using the PRODA database management system (Conceptual Software, Inc, Houston, TX). Multivariate Cox proportional hazards models were fitted to assess whether elevated levels of biomarkers, clinical variables, or pathologic parameters were associated with DMFS, CSS, and OS. Separate models were fitted to assess the effect of cytoplasmic BAG-1 in the total patients sample, those with negative axillary lymph nodes,

and those with positive axillary lymph nodes. All multivariate models contained candidate variables including nuclear BAG-1, Bcl-2, ER, and stage. OS, CSS, and DMFS curves were calculated by the life-table method, with differences between the curves tested by the Mantel-Haenszel statistics test. Variables were included in the model using a stepwise variable selection procedure. A two-sided *P* value of  $\leq .05$  was considered statistically significant.

#### RESULTS

##### *BAG-1 Expression Patterns in Normal Breast Epithelium (NBE) and Malignant Breast Cancers*

BAG-1 immunostaining was compared in the invasive components of breast tumors and adjacent normal breast epithelium present in the same breast cancer biopsy sections. The monoclonal BAG-1 antibody demonstrated specific immunoreactivity in both NBE and invasive breast cancers as demonstrated in Fig 2. Depending on the particular tissue specimen, BAG-1 immunoreactivity in normal and neoplastic mammary cells was found in cytosol, nucleus, or both, consistent with prior studies of BAG-1 immunolocalization in normal tissues.<sup>28</sup> In NBE, BAG-1 immunostaining was generally weak (Fig 2A and B). When present, BAG-1 immunostaining was typically found in the nuclei of NBE cells but not the cytosol, with 25 (28%) of 88 NBE specimens having H-scores  $\geq 150$  for nuclear immunostaining (Table 2). In contrast, although the frequency of invasive cancers with high levels of nuclear BAG-1 immunostaining was not significantly different from NBE (Table 2), cytosolic BAG-1 immunostaining was clearly elevated in many tumors compared with adjacent NBE. Analysis of the immunostaining scores (H-scores) for cytosolic BAG-1

## BAG-1 Upregulation in Breast Cancer

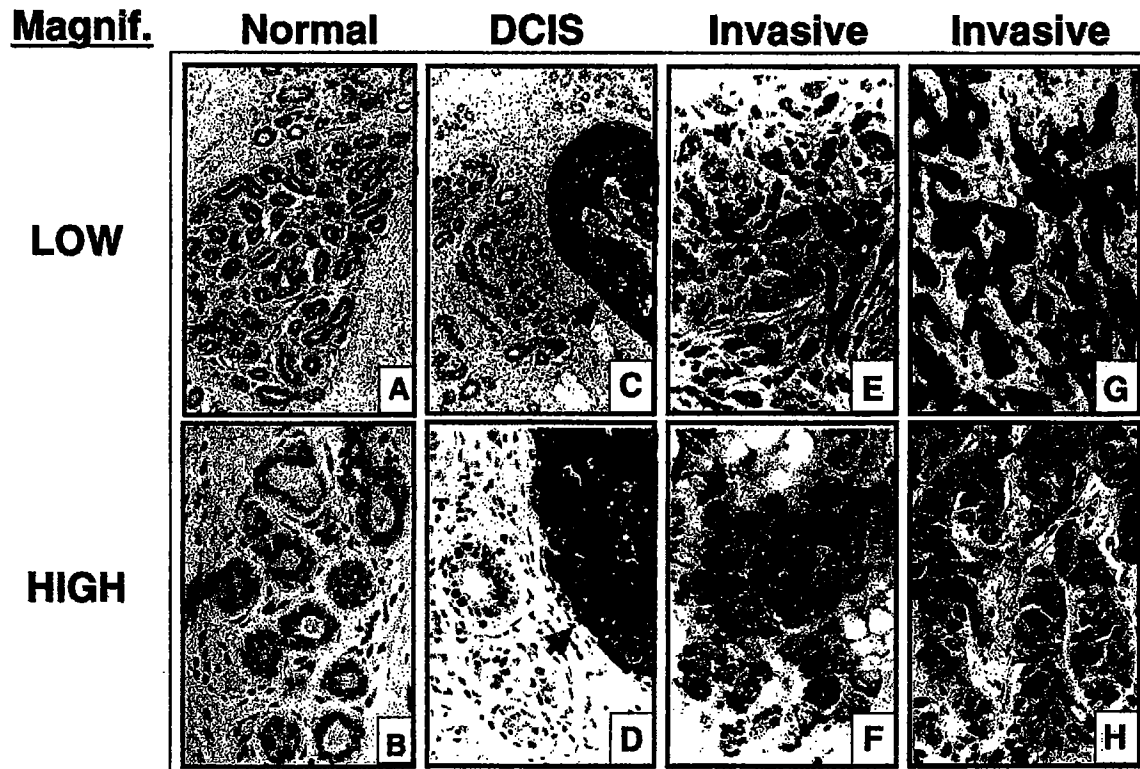


Fig 2. BAG-1 Immunostaining. Abbreviation: DCIS, ductal carcinomas in situ.

demonstrated a bimodal distribution, with invasive cancers having H-scores  $\geq 150$  representing a subgroup having clearly higher levels of this protein (Fig 1). Among the 122 tumor biopsies analyzed, 79 (65%) had invasive cancer

Table 2. Expression of BAG-1 and Bcl-2 in Normal Breast Epithelium and Invasive Cancer Components

Protein	NBE		Cancer		P
	No.	%	No.	%	
BAG-1, cytosolic	10/88	11	79/122	65	< .001
BAG-1, nuclear	25/88	28	28/122	23	NS
Bcl-2	18/106	17	70/106	66	< .001

NOTE. NBE and invasive cancer components were compared with regards to immunointensity. Specimens having an H-score (intensity  $\times$  distribution)  $\geq 150$  for BAG-1 and  $\geq 180$  for Bcl-2 protein positivity were considered positive. Data represent the fraction of immunopositive specimens as determined by mean H-score system. Statistical significance was determined by Fisher  $\chi^2$  analysis.

Abbreviations: NBE, normal breast epithelium; NS, not significant.

components that contained high-level (H-score  $\geq 150$ ) cytosolic BAG-1 immunostaining (Table 2), compared with only 10 (11%) of 88 of NBE specimens ( $P < .001$ ). Thus, roughly two thirds of early-stage breast cancers contained elevated levels of BAG-1 protein in their cytosol, suggesting that upregulation of cytosolic BAG-1 represents a tumor-specific event for a subset of these malignancies. Some of the same tumor specimens also contained histologically evident ductal carcinomas in situ (DCIS) lesions. High levels of cytoplasmic and nuclear BAG-1 immunostaining were present in nine (64%) of 14 and seven (50%) of 14 DCIS specimens, respectively, suggesting that upregulation of BAG-1 can occur as a relatively early event in tumorigenesis (Fig 2C and D; data not shown).

### Bcl-2 Expression in Breast Cancer

It has been shown that the antiapoptotic protein Bcl-2 becomes upregulated during the transition from benign to

malignant epithelium in the breast (reviewed in<sup>37</sup>). Overexpression of the Bcl-2 protein has been correlated with improved survival in both node-negative and node-positive breast cancer patients.<sup>18,19</sup> Analysis of Bcl-2 immunoscores revealed a bimodal distribution, with invasive cancers having H-scores  $\geq 180$  representing a subgroup with distinctly higher levels of this antiapoptotic protein (Fig 1). Of the 106 tumor specimens successfully stained for Bcl-2, 70 (66%) had H-scores  $\geq 180$  (Table 2). Comparison of Bcl-2 and BAG-1 immunostaining data revealed that 62 (82%) of 76 breast tumors had overexpression of both proteins, demonstrating a statistically significant positive correlation of cytosolic BAG-1 immunostaining with Bcl-2 expression ( $P = .0005$ ). Thus, expression of BAG-1 and Bcl-2 may be coregulated to some extent in early-stage invasive breast cancers.

#### Univariate Analysis of BAG-1

Life-table analysis revealed that elevated levels of BAG-1 were statistically significantly associated with longer DMFS ( $P < .001$ ) and OS ( $P < .001$ ) (Fig 3, Table

3). The 10-year DMFS for breast cancer patients with BAG-1 protein overexpression was 79% compared with 34% for those whose tumors scored low for BAG-1 immunoreactivity (Fig 3, Table 3). Likewise, the 10-year OS for women with high BAG-1 protein levels was 82% compared with 42% for patients with breast tumors having low levels of this protein (Fig 3, Table 3). There were 79 (65%) of 122 of breast tumors that were BAG-1 positive and 43 (35%) of 122 BAG-1 negative that were used for the analysis of OS and DMFS (Table 2, Fig 3). The 10-year OS for all 122 breast cancer patients in the study was 68% (data not shown), compared with 82% for patients with early-stage breast cancer that overexpresses the BAG-1 protein ( $P = .01$ ) (Table 3). A strong relationship between the overexpression of cytoplasmic BAG-1 protein and improved survival in breast cancer patients was also detected using several alternative H-score cutoffs, including 25 and 75, in addition to the optimized cutoff of 150 (data not shown). We also found that elevated levels of BAG-1 were associated with improved DMFS ( $P < .01$ ) and OS ( $P < .01$ )

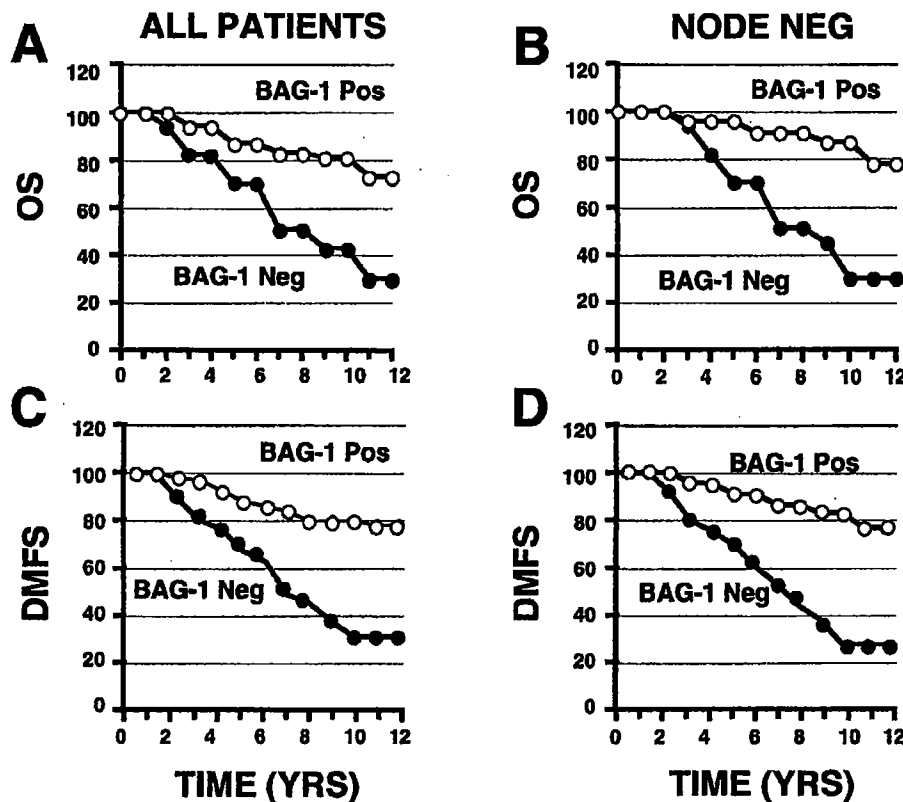


Fig 3. High BAG-1 protein levels associated with improved OS. Abbreviations: Neg, negative; Pos, positive; YRS, years; OS, overall survival; DMFS, distant metastasis-free survival.

when examining levels of intensity of BAG-1 immunoreactivity (intensity  $\geq 2$ ; data not shown).

Because the identification of prognostic biomarkers has a potentially greater role in axillary lymph node-negative breast cancer patients, we performed subgroup analysis examining the importance of BAG-1 cytoplasmic expression in 63 women who underwent axillary lymph node dissection (Table 1). In 49 breast cancer patients with pathologically determined negative axillary lymph nodes, the 10-year OS for women with cytoplasmic expression of BAG-1 was 87% compared with 30% for those patients with low levels of BAG-1 protein ( $P = .001$ ) (Table 3). We also found that elevated levels of BAG-1 protein were associated with significantly improved DMFS and CSS in breast cancer patients whose axillary lymph nodes were not involved with breast cancer ( $P = .002$ ) (Table 3). Although the expression of Bcl-2 often correlates with ER and PR positivity,<sup>18,19,34</sup> we found no correlations between the levels of nuclear or cytoplasmic BAG-1 protein and ER or PR positivity using several different monoclonal antibodies in breast cancer specimens used for this study (data not shown).

**Table 3. Comparison of Biomarkers With 10-Year Survival Parameters.**

Marker	OS		DMFS		CSS	
	%	P	%	P	%	P
Cytoplasmic BAG-1						
Neg	42		34		48	
Pos	82	< .001	79	< .001	84	< .001
Cytoplasmic BAG-1, lymph node negative						
Neg	30		29		37	
Pos	87	.001	81	.002	87	.002
Nuclear BAG-1						
Neg	71		64		70	
Pos	79	NS	71	NS	86	.05
Bcl-2						
Neg	40		43		40	
Pos	86	< .001	84	< .001	92	< .001
p53						
Neg	69		70		78	
Pos	55	NS	50	NS	55	.05
HER2/Neu						
Neg	64		62		70	
Pos	73	NS	65	NS	73	NS
ER						
Neg	65		66		69	
Pos	70	NS	74	NS	76	NS
PR						
Neg	66		62		72	
Pos	62	NS	59	NS	68	NS

NOTE. The 10-year survival rates were estimated by the life-table method and differences determined by Mantel-Haenszel. Immunostaining was analyzed by the H-scoring method, using cutoffs of 150, 180, 50, and 75 for BAG-1, Bcl-2, p53, and ER, respectively.<sup>17</sup>

Abbreviations: Neg, negative; Pos, positive.

#### Univariate Analysis of Bcl-2

Among the other biomarkers evaluated, Bcl-2 was statistically significant in univariate analysis as a predictor of CSS ( $P < .001$ ), DMFS ( $P < .001$ ), and OS ( $P < .001$ ) (Table 3). The 10-year OS in early-stage breast cancer patients with high levels of Bcl-2 protein immunoreactivity was 86% compared with 40% for women with breast tumors having low levels of the Bcl-2 protein ( $P < .001$ ) (Fig 3, Table 3). The statistically significant relationship with Bcl-2 expression and improved survival was also demonstrated at alternative H-scores, including 25 and 75 (not shown). Our findings are consistent with prior studies demonstrating an important relationship between expression of Bcl-2 protein and improved survival (reviewed in<sup>38,39</sup>). We also found that Bcl-2 positive breast tumors were more likely to be ER positive ( $P = .01$ ) but were not associated with PR positivity ( $P =$  not significant), consistent with prior reports (reviewed in<sup>38,39</sup>).

#### Correlations of Other Markers and Survival

In the same cohort of patients, immunohistochemical overexpression of mutant p53 protein was associated with poor CSS ( $P = .05$ ) but not OS or DMFS in univariate analysis (Table 3). Although ER, PR, and HER2/Neu were not of statistical significance as far as predicting survival for this cohort of patients, we did observe a trend to better survival rates in women with breast tumors that expressed ER (Table 3). Among clinical, pathologic, and molecular variables (age, tumor size, stage, tumor histology, PR, p53, HER2/Neu), only stage (I v II) was significant in univariate analysis for predicting OS ( $P = .006$ ; data not shown). There were 38 (31%) of 122 breast cancer patients treated with adjuvant chemotherapy or hormonal therapy, and no significant correlation was seen with respect to OS, DMFS, or CSS in this limited cohort of patients using univariate analysis (data not shown).

#### Multivariate Analysis of BAG-1 and Bcl-2

In multivariate analysis using Cox proportional hazards models with variables including BAG-1, Bcl-2, ER, and stage, only the expression of cytoplasmic BAG-1 protein was a statistically significant predictor of OS ( $P = .01$ ), DMFS ( $P = .005$ ), and CSS ( $P = .008$ ) in all 122 breast cancer patients (Table 4). Elevated BAG-1 protein was also statistically significant in a separate multivariate analysis in women with negative axillary lymph nodes for OS ( $P = .001$ ), DMFS ( $P = .005$ ), and CSS ( $P = .002$ ) (Table 4). We did not find any significant relationships between BAG-1 protein and survival in women with positive axillary lymph node, which maybe related to the small size of this sub-

Table 4. Multivariate Analysis of Prognostic Factors With 10-Year Survival Parameters

Marker	OS P	DMFS P	CSS P
Cytoplasmic BAG-1			
All breast cancer patients	.01	.005	.008
Lymph node-negative breast cancer patients	.001	.005	.002
Lymph node-positive breast cancer patients	NS	NS	NS
Nuclear BAG-1, all breast cancer patients	NS	NS	NS
Bcl-2, all breast cancer patients	.07	.11	.007
ER, all breast cancer patients	NS	NS	NS
Stage, all breast cancer patients	.04	.02	.05

NOTE. Data were dichotomized into high versus low groups using H-scores of  $\geq 150$ ,  $\geq 180$ ,  $\geq 75$ , and  $\geq 25$  for BAG-1, Bcl-2, and ER, respectively. Staging was performed using American Joint Committee on Cancer criteria. Multivariate analysis was performed using a Cox proportional hazards regression model, which included BAG-1, Bcl-2, ER, and stage in three multivariate models including all breast cancer patients, lymph node-negative breast cancer patients, and lymph node-positive breast cancer patients.

group. The expression of Bcl-2 was an independent predictor for CSS ( $P = .007$ ) but not DMFS or OS (Table 4). Use of alternative H-scores for BAG-1 and Bcl-2 as described above did not change these conclusions (not shown). There were 38 (31%) of 122 of patients who received systemic therapy, and multivariate analysis revealed that elevated levels of BAG-1 remained a strong predictor of OS when these patients were removed from the analysis, which suggests these findings are not dependent on adjuvant therapy ( $P = .02$ ; data not shown). All other biomarkers including ER and nuclear BAG-1 failed to reach statistical significance. In multivariate analysis of all 122 patients, stage was significant for DMFS ( $P = .02$ ) and OS ( $P = .04$ ) (Table 4).

## DISCUSSION

This is the first study to address the potential prognostic significance of BAG-1 using highly specific immunohistochemical reagents in early-stage breast cancer patients treated uniformly with breast-conserving therapy. The data presented here demonstrate that overexpression of BAG-1 correlates with improved survival in early-stage breast cancer patients, including patients with pathologically-documented negative axillary lymph node biopsies. The *bag-1* gene encodes two major proteins, including BAG-1 and a longer nuclear-targeted isoform BAG-1L.<sup>28,29</sup> Although BAG-1 is preferentially found in the cytosol, the BAG-1L protein is exclusively nuclear.<sup>28,29</sup> However, the shorter BAG-1 protein can also be found in the nucleus under some circumstances, probably as a result of its association with

other proteins that enter nuclei.<sup>24,28</sup> Interestingly, although both normal and malignant mammary epithelial cells sometimes contained prominent nuclear BAG-1 immunoreactivity, cytosolic BAG-1 immunostaining was rarely present at high levels in normal mammary epithelium, whereas cytosolic BAG-1 was clearly elevated in 66% of the invasive breast carcinomas evaluated when compared with normal mammary epithelium.

A recent report of BAG-1 immunostaining in a diverse cohort of breast cancer patients, including women with early- and late-stage disease undergoing a wide variety of therapies, demonstrated upregulation of both nuclear and cytoplasmic BAG-1 immunostaining in 77% of breast cancers studied and increases in cytoplasmic staining without concomitant nuclear staining in 57% of patient tumor specimens.<sup>33</sup> Thus, although that recent study is in agreement with our observations concerning upregulation of cytosolic BAG-1 immunostaining, our findings differ from those of Tang et al<sup>33</sup> with respect to nuclear BAG-1 immunoreactivity. Moreover, Tang et al found that high levels of nuclear BAG-1 immunostaining were associated with shorter, rather than longer, disease-free and overall survival in their collection of heterogeneous patient tumor specimens. Several factors could contribute to differences in the results obtained recently by others and those reported here. For example, the study by Tang et al used a polyclonal anti-BAG-1 antibody that recognizes several isoforms of the BAG-1 protein. The anti-BAG-1 monoclonal antibody chosen for our study, however, has been confirmed to lack cross-reactivity with other BAG-family proteins, including BAGL2, BAGL3, BAGL4, and BAGL5.<sup>40</sup> In contrast, the reactivity of polyclonal antisera with other members of the BAG family has not been addressed. Thus, differences in antibody reagents may play a role in the differences observed. In addition, the cohort of patients examined in our study consisted entirely of early-stage breast cancer patients (stages I and II), whereas the recent report by others included 27% of patients with either metastatic disease or unknown stage.<sup>33</sup> Finally, we correlated nuclear and cytosolic BAG-1 immunoreactivity separately with patient outcome, whereas the method used by Tang et al combined nuclear and cytosolic staining data.

The BAG-1 protein has been shown to form complexes with and modulate the activities of a variety of proteins involved in cell proliferation, survival, and differentiation, including the antiapoptotic protein Bcl-2, the kinase Raf-1, the tyrosine kinase growth factor receptors for platelet-derived growth factor and hepatocyte growth factor, the growth regulator Siah-1, and retinoic acid receptors.<sup>4,21,22,24,25,27</sup> In every instance evaluated so far in cultured cells, overexpression of BAG-1 has been associated with enhanced cell proliferation and survival. Thus, contrary to expectations, higher levels of



cytosolic BAG-1 immunostaining were paradoxically associated with longer OS in the cohort of early-stage breast cancer patients used for this study. The molecular basis for this observation can only be speculated, but it may be related to either (a) the recent realization that BAG-1 is only one member of a family of at least five similar Hsc70/Hsp70-binding proteins that potentially compete for binding to these molecular chaperones<sup>40</sup> or (b) the role of Hip and related cochaperones that also compete with BAG-1 for binding to Hsc70/Hsp70 and that have opposing effects compared with BAG-1 on the Hsc70/Hsp70-mediated peptide refolding cycle.<sup>30,40,42-44</sup> Thus, the ratio of BAG-1 protein relative to other BAG-family members or relative to Hip and its related cochaperones may be the ultimate arbiter of biologic responses, rather than the absolute levels of BAG-1 alone.<sup>45</sup>

The utility of Bcl-2 as a marker of favorable outcome in breast cancer has been established by prior studies, which have included cohorts of breast cancer patients with node-negative or node-positive disease.<sup>18,19</sup> In agreement with these studies, we also found that overexpression of Bcl-2 protein in breast cancer specimens was associated with improved survival characteristics in univariate but not multivariate analysis. Given that Bcl-2 is a potent blocker of apoptosis, higher levels of this antiapoptotic protein would not be expected to correlate with better clinical outcome. However, Bcl-2 is also an antiproliferative protein, at least in some cellular contexts,<sup>46,47</sup> and its antiapoptotic and antiproliferative functions are separable.<sup>48</sup> Moreover, the activity of Bcl-2 can be either positively or negatively modulated by phosphorylation,<sup>49,50</sup> which is not detected by immunohistochemical assays. Thus, the functional status of the Bcl-2 protein in breast cancers remains undefined. Furthermore, ratios of Bcl-2 relative to other members of the Bcl-2 family may play a role in dictating the ultimate phenotypes conferred by this protein, because (a) high levels of Bcl-2 have even been associated with enhanced rather than suppressed sensitivity to apoptosis in some

cellular contexts<sup>51</sup> and (b) the ratio of Bcl-2 to Bax can influence the antiproliferative effects of Bcl-2.<sup>47</sup>

Approximately 30% to 40% of patients with apparently localized breast cancer probably have micrometastatic disease, which is clinically undetectable at the time of diagnosis and which accounts for most instances of distant relapses and disease-related deaths.<sup>1,3</sup> Predictive biomarkers are greatly needed that can help guide clinicians and patients in treatment-related decisions about the necessity (or lack thereof) for adjuvant chemotherapy, hormonal therapy, and new treatments as they become available. The results reported herein indicate that a large proportion of early-stage primary breast cancers arise through a pathway that includes upregulation of BAG-1 protein expression, in agreement with a recent report.<sup>33</sup> The axillary lymph node-negative patients whose breast tumors contain elevated levels of cytosolic BAG-1 protein were found to be more likely to enjoy long-term survival and freedom from distant metastases, compared with those with BAG-1 negative tumors. Moreover, these findings are independent of systemic therapy. Because many of the patients did not undergo axillary dissection or were axillary lymph node-negative, the predictive value of BAG-1 protein in node-positive breast cancer patients remains to be determined and the importance of BAG-1 in predicting survival in lymph node-negative breast cancer patients will now need to be confirmed. Additional studies that seek to establish optimal methods for quantifying expression of specific isoforms of BAG-1 and that involve larger cohorts of patients in prospective trials are needed to firmly establish the overall prognostic utility of BAG-1 testing for women with early-stage breast cancer.

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